



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/56, 1/21, 9/28 A23K 3/03	A1	(11) International Publication Number: WO 92/19744 (43) International Publication Date: 12 November 1992 (12.11.92)
(21) International Application Number: PCT/FI92/00138 (22) International Filing Date: 4 May 1992 (04.05.92) (30) Priority data: 695,214 6 May 1991 (06.05.91) US (71) Applicant: VALIO, FINNISH CO-OPERATIVE DAIRIES' ASSOCIATION [FI/FI]; PL 390, SF-00101 Helsinki (FI). (72) Inventors: KOIVULA, Teija ; Porvoonkatu 16 A 36, SF-00510 Helsinki (FI). SIBAKOV, Mervi ; Ylipalontie 21 C, SF-00670 Helsinki (). PALVA, Ilkka ; Vålitalontie 87 A, SF-00660 Helsinki (FI). (74) Agent: OY JALO ANT-WUORINEN AB; Laivuririnne 2 A, SF-00120 Helsinki (FI).		(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), NO, SE (European patent). Published <i>With international search report.</i>
(54) Title: ACID α -AMYLASE (57) Abstract <p>The invention is related to the recombinant production of acid α-amylase, DNA sequences encoding such acid α-amylase, vectors containing such DNA, and the expression of such DNA in a recombinant host cell. The invention is further related to a method of feed and fodder preservation using the hosts of the invention.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

-1-

Acid α -AmylaseField of the Invention

The present invention is directed to purified genetic and protein sequences encoding the acid α -amylase protein of *B. acidocaldarius*, recombinant constructs and hosts transformed therewith that are capable of expressing such sequences, and the use of such genetic sequences for the expression of acidic α -amylase, and the use of the recombinantly-produced acid α -amylase protein for industrial and agricultural applications such as, for example, starch liquefaction, and silage preparation.

Brief Description of the Background Art

Lactobacillus and *Bacillus* strains offer many potential advantages in the production of cloned gene products, as compared with *Escherichia coli*. First, they are non-pathogenic and do not synthesize endotoxins. Second, many of the gene products are secreted into the growth medium, in contrast to *E. coli*, which retains most of the proteins due to the presence of an outer membrane. Third, they have been widely used for production of enzymes in large-scale fermentation processes. While *Lactobacillus* is not an industrial enzyme producer, it is highly used in the food and animal feed processing industry.

One important highly desirable exoenzyme secreted in large amounts by *Bacilli* is α -amylase. α -Amylase is used in a variety of industrial applications, for

-2-

example, starch liquefaction. However, the industrial use of this enzyme is limited in many cases by its sensitivity to acidic conditions. That is, most forms of the enzyme are not enzymatically stable at acidic pH's.

At least four acidophilic and acid-stable α -amylase isozymes have been reported in different strains of *B. acidocaldarius* (Kanno, M., *Agric. Biol. Chem.* 50:23-31 (1986); Buonocore, V. et al., *J. Bacteriol.* 128:515 (1976); Boyer, E.W. et al., *Starch/Stärke* 31:166 (1979); and Uchino, F., *Agric. Biol. Chem.* 46:7 (1982). An "acidic" α -amylase is characterized as being relatively stable to heat under acidic conditions. For example, the acid α -amylase's produced by *Bacillus acidocaldarius* strain A2 retains more than 79-90% of its activity after 30 min of incubation at pH 2.0 (70°C) and at pH 4.5 (90°C) in the absence of substrate (Kanno, M., *Agric. Biol. Chem.* 50:23-31 (1986).

α -Amylase has also been isolated from *B. amyloliquefaciens* (Ingle et al., *Adv. Appl. Microbiol.* 24:257-278 (1987)). This enzyme has an M_r -value of about 50,000 daltons and has been sequenced (Takkinen et al., *J. Biol. Chem.* 258: 1007-1013 (1983); Chung et al., *Biochem. J.* 185:387-395 (1980)). The expression of this enzyme in *B. subtilis* has been reported ("Expression and Regulation of the *Bacillus amyloliquefaciens* α -amylase gene in *B. subtilis*," P.Kallio, Ph.D. dissertation, University of Helsinki, 1987).

Acidic amylase sequences are especially desirable for industrial purposes, where pH values may fall (meaning the medium becomes more acidic) during a

desired reaction due to the natural catalytic action of the enzymes therein. For example, a process strain that possesses an acid α -amylase activity would be valuable for the *Lactobacillus* strains as lactic acid bacteria are widely used for preservation of many starch containing raw materials (e.g., cereal grains, edible roots and crop residues).

Although such approaches and applications have been highly desired in the art (*Applied Environment. Microbiol.* 55:2130-2137 (1989)), they have not been possible to obtain. The main obstacle was the lack of an α -amylase enzyme that is not inactivated by the low growth pH of *Lactobacillus*. So far, no genes of bacterial origin, coding for an acid α -amylase enzyme, have been characterized and the isolation of large quantities of such acid α -amylases from natural sources is relatively expensive. This has precluded the wide-spread adoption of such enzymes in industry. Accordingly, a need exists for a cost-efficient recombinant source of a highly stable acid α -amylase.

SUMMARY OF THE INVENTION

Recognizing the importance to the feed industry of a host that would be capable of providing an acid-stable α -amylase activity during the food preservation period, and cognizant of the lack of hosts capable of providing such enzymatic activity in the currently utilized processes, the inventors have investigated the properties of the acid α -amylase from *B. acidocaldarius*. The inventors desired to generate a new *Lactobacillus* host, such host having a novel

-4-

property that would be advantageous in the food and feed industry.

These efforts have culminated in the development of novel recombinant forms of acid α -amylase, hosts expressing such acid α -amylase, and highly improved processes for the preservation of food utilizing such hosts.

According to the invention, there are first provided a polynucleotide, such polynucleotide providing the coding sequence of an acid α -amylase.

According to the invention, there are also provided recombinant vectors, such vectors providing a host expressible form of acid α -amylase.

According to the invention, there are further provided host cells transformed with polynucleotides and/or vectors that are capable of expressing recombinant forms of acid α -amylase.

According to the invention, there are further provided methods for producing the genetically engineered or recombinant protein acid α -amylase of the invention using such hosts.

According to the invention, there are further provided methods for the utilization of the acid α -amylase enzyme of the invention.

DESCRIPTION OF THE FIGURES

Figure 1. SDS-PAGE of the $(\text{NH}_4)_2\text{SO}_4$ precipitated proteins. Lanes 1 and 2 represent Coomassie Brilliant Blue R stained gel and zymogram, respectively. The arrows show the positions of the bands corresponding amylase activity. Molecular weight markers (Pharmacia) are indicated on the left.

-5-

Figure 2. Effect of pH on the activity of *B. acidocaldarius* amylase(s). Enzyme activity in 100 μ l of the 0.5 M sodium acetate, pH 5.0 eluted proteins of *B. acidocaldarius* cultures was measured using Phadebas[®] amylase test at 60°C in 0.1 M citric acid, 0.2 M Na_2HPO_4 .

Figure 3. The nucleotide sequence of the *B. acidocaldarius* acid α -amylase gene. The putative -35 and -10 regions (bases 126 and 148, respectively) of the amylase promoter are underlined. The potential signal sequence cleavage site (base 273) is indicated by an arrow. The N-terminal sequence (base 1594) of the 90 kd protein is underlined with a dotted line. The two alternative N-termini of the signal peptide are indicated by an asterisk.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

In the description that follows, a number of terms used in recombinant DNA (rDNA) technology are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Process strain. A "process strain" is a bacterial strain that causes or contributes to a desired change in a biotechnical process.

Biotechnical process. A biotechnical process is a process that depends upon the production of bacteria or biomass for the achievement of a desired

-6-

chemical transformation of the process medium, such bacteria or biomass providing a component or environment necessary for such conversion.

Fodder. "Fodder" is anything fed to domesticated animals, and especially, coarse food for cattle, horses or sheep.

Forage. "Forage" is food for domesticated animals that is taken by browsing or grazing.

Silage. "Silage" is fodder converted into succulent feed for livestock through processes of anaerobic acid fermentation (as occurs in a silo).

Gene. A DNA sequence containing a template for a RNA polymerase. The RNA transcribed from a gene may or may not code for a protein. RNA that codes for a protein is termed messenger RNA (mRNA).

A "complementary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA and from which intervening sequences (introns) have been removed.

Genetic sequence. As used herein, the term "genetic sequences" is intended to refer to a nucleic acid molecule (preferably DNA). Accordingly, "acid α -amylase genetic sequences" are DNA or RNA sequences that possess a nucleotide sequence that encodes the acid α -amylase protein.

Promoter. The term "promoter" as used herein refers to a module or group of modules which, at a minimum, provides a binding site or initiation site for RNA polymerase action that is sufficient to initiate transcription of the operably linked in a desired host. A promoter is generally composed of multiple operably linked genetic elements termed herein "modules."

-7-

Promoter Module. The term "module" as in "promoter module" refers to a genetic transcriptional regulatory element which provides some measure of control over the transcription of operably linked coding sequences or other operably linked modules.

Each module in a promoter can convey a specific piece of regulatory information to the host cell's transcriptional machinery. At least one module in a promoter functions to position the start site for RNA synthesis. Other promoter modules regulate the frequency of transcriptional initiation. Typically, modules which regulate the frequency of transcriptional initiation are located upstream of (i.e., 5' to) the transcriptional start site, although such modules may also be found downstream of (i.e., 3' to) the start site.

The term "target module," as used herein, refers to a transcriptional regulatory element which confers the ability to respond to enhancer gene activity (i.e., such as the protein or peptide encoded by an enhancer gene) on a promoter which otherwise would not respond, or would respond less efficiently, to such enhancer gene activity.

The term "initiation module" refers to a promoter module which is required to initiate transcription of operably linked genes with RNA polymerase. In prokaryotic promoters, initiation modules are usually located at about -10 and -35 nucleotides from the start site of transcription.

By "hybrid promoter" is meant a promoter in which an initiation module is operably linked to a heterologous target module. A target module which is heterologous to an initiation module is a target

module which is not found naturally operably linked to this initiation module in the host cell.

Operable linkage. An "operable linkage" is a linkage in which a sequence is connected to another sequence (or sequences) in such a way as to be capable of altering the functioning of the sequence (or sequences). For example, a protein encoding sequence which is operably linked to the hybrid promoter of the invention places expression of the protein encoding sequence under the influence or control of the regulatory sequence. Two DNA sequences (such as a protein encoding sequence and a promoter region sequence linked to the 5' end of the encoding sequence) are said to be operably linked if induction of promoter function results in the transcription of the protein encoding sequence mRNA and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the mRNA or protein. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence.

Cloning vector. A "cloning vector" is a plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vector may

-9-

further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, are erythromycin and kanamycin resistance. The term "vehicle" is sometimes used for "vector."

Expression vector. An "expression vector" is a vector similar to a cloning vector but is capable of expressing a structural gene which has been cloned into the expression vector; after transformation of the expression vector into a host. In an expression vector, the cloned structural gene (any coding sequence of interest) is placed under the control of (i.e., operably linked to) certain control sequences which allow such gene to be expressed in a specific host. In the expression vector of the invention, a desired structural gene is operably linked to the hybrid promoter of the invention. Expression control sequences will vary, and may additionally contain transcriptional elements such as termination sequences and/or translational elements such as initiation and termination sites.

The expression vectors of the invention may further provide, in an expression cassette other than the one providing the hybrid promoters of the invention, sequences encoding a desired enhancer gene. In a preferred embodiment, such enhancer gene would be the enhancer gene which encodes the protein which regulates the target module of the hybrid promoter.

Functional Derivative. A "functional derivative" of a molecule, such as a nucleic acid or protein, is a molecule which has been derived from a native molecule, and which possesses a biological activity (either functional or structural) that is substan-

-10-

tially similar to a biological activity of the native molecule, but not identical to the native molecule. By a functional derivative of a nucleic acid sequence (herein the "second sequence") that encodes a protein (herein a "first" sequence) is meant (1) a nucleic acid sequence that does not possess the exact same nucleotide sequence as the first sequence but which encodes the same amino acid sequence; and, a nucleic acid sequence that does not encode the same amino acid sequence but which encodes a protein with biochemical properties (such as, for example, enzymatic stability in acidic conditions) equivalent to (i.e., the same as or slightly different from) the protein encoded by the first nucleic acid sequence.

A functional derivative of a protein is a protein that retains a desired function of the "parent" protein from which it is derived. A functional derivative of a protein may or may not contain post-translational modifications, such as covalently linked carbohydrate, depending on the necessity of such modifications for the performance of a specific function. The term "functional derivative" is intended to include the "fragments," "variants," or "chemical derivatives" of a molecule.

As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are

-11-

disclosed in *Remington's Pharmaceutical Sciences* (1980). Procedures for coupling such moieties to a molecule are well known in the art.

Fragment. A "fragment" of a molecule such as a nucleic acid or protein is meant to refer to a molecule which contains a portion of the complete sequence of the native molecule.

Variant. A "variant" of a molecule such as a nucleic acid or protein is meant to refer to a molecule substantially similar in structure and biological activity to either the entire molecule, or to a fragment thereof, but not identical to such molecule or fragment thereof. A variant is not necessarily derived from the native molecule itself. A gene allele is an example of what is meant by a variant nucleic acid sequence and an enzymatic isozyme is an example of what is meant by a variant enzyme sequence. Thus, provided that two molecules possess a similar biological activity or function that characterizes molecules as a 'family', they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the sequence of nucleic acid (or amino acid residues) is not identical, or if the synthesis of one of the variants did not derive from the other. Thus the invention intends to include all variants (genomic alleles and enzymatic isozymes) of the acid α -amylase of the invention.

II. Genetic Engineering of Acid α -amylase

Prior to the invention, the cloning of the acid amylase gene was not thought to be possible due to the lack of cross-reacting antibodies, the lack of sufficient protein to obtain such antibodies, the lack of a reliable probe and the lack of protein sequence data from which to derive such probe.

The available antisera or gene probes of other known bacterial amylases (for example, those of *B. amyloliquefaciens* strain A2 as described by Kanno, M. Agric. Biol. Chem. 50:23-31 (1986), *B. licheniformis* as described by Siato, N., Arch. Biochem. Biophys. 155:290 (1973) and *B. subtilis*) does not provide any crossreactivity or positive hybridization when tested with material derived from *B. acidocaldarius*. When the amylase preparation from *B. amyloliquefaciens* was analyzed by zymography, it revealed an amylase band of molecular weight 150,000-200,000 daltons. This is much higher than previously reported for other bacterial amylases. Due to the very low initial yield and large size of the acid amylase, it was not possible to determine the N-terminal sequence of this protein by standard protein chemistry methods in order to obtain an oligonucleotide probe.

Thus it was necessary to identify clones to the *B. acidocaldarius* acid amylase without having available (1) antibodies that cross-reacted with the protein or (2) Protein or DNA sequence information upon which to design a oligonucleotide probe.

To solve this problem, a new method for the identification of acid amylase protein and clones was developed. A 90,000 dalton peptide was found in

-13-

protein extracts of *B. acidocaldarius*, that had a very low specific amylase activity. An assumption was made that this peptide would be sufficiently related to the large acid amylase protein to allow selection of clones to the acid amylase protein, using probes designed from the sequence of the smaller peptide. This was not a risk-free assumption as the low level "amylase" activity displayed by the 90,000 dalton peptide could easily have been a side activity of a different type of enzyme, and not related to the acid amylase enzyme of the invention *per se*. Thus, there was no way of confirming that the assumption upon which the cloning of the enzyme of the invention was based was correct until the entire cloning and sequencing was complete.

The process for genetically engineering the acid α -amylase sequences of the invention is facilitated through the cloning of genetic sequences which are capable of providing specific protein encoding sequences. Genetic sequences which are capable of providing protein encoding sequences may be derived from genomic DNA, synthetic DNA, cloned DNA and combinations thereof. The preferred species source of the acid α -amylase of the invention is *B. acidocaldarius*, although any source of an acid α -amylase may be used.

Genetic (protein encoding) genomic DNA will not contain introns in prokaryotes, although it may contain spacers between transcriptional units. As outlined below, such genomic DNA may be obtained in association with the 5' promoter region and/or the 3' transcriptional termination region if desired. Further, such genomic DNA may be obtained in

association with the genetic sequences which encode a 5' non-translated region of the desired mRNA and/or with the genetic sequences which encode the 3' non-translated region. To the extent that a host cell can recognize the transcriptional and/or translational regulatory signals associated with the expression of the mRNA and protein, and to the extent that such signals do not impede the expression of the recombinant acidic α -amylase of the invention, then the 5' and/or 3' non-transcribed regions of the native gene, and/or, the 5' and/or 3' non-translated regions of the mRNA, may be retained and employed for transcriptional and translational regulation.

The coding sequences for the acid α -amylase of the invention is not rearranged by the native bacterial host prior to expression in such host. To obtain coding sequences for proteins whose genes are not rearranged prior to expression, genomic DNA can be extracted and purified from any cell of any host which carries the coding sequence, whether or not the cell expresses the protein. Such extraction of genomic DNA can be performed by means well known in the art (for example, see *Guide to Molecular Cloning Techniques*, S.L. Berger et al., eds., Academic Press (1987)).

Alternatively, nucleic acid sequences that encode a desired protein can also be obtained by DNA transcribed from mRNA specific for that protein. mRNA can be isolated from any cell which produces or expresses the protein of interest and used to produce cDNA by means well known in the art (for example, see *Guide to Molecular Cloning Techniques*, S.L. Berger et al., eds., Academic Press (1987)). Preferably, the mRNA preparation used will be enriched in mRNA coding

for the desired protein, either naturally, by isolation from a cells which are producing large amounts of the protein, or *in vitro*, by techniques commonly used to enrich mRNA preparations for specific sequences, such as for example, gel electrophoresis, sucrose gradient centrifugation.

To prepare DNA for cloning into a cloning vector or an expression vector, a suitable DNA preparation (either genomic DNA or cDNA) is randomly sheared or enzymatically cleaved, respectively. Such DNA can then be ligated into appropriate vectors to form a recombinant gene (either genomic or cDNA) library.

A DNA sequence encoding a protein of interest or its functional derivatives may be inserted into a cloning vector or an expression vector in accordance with conventional techniques, including blunt-ending or staggered-ending termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed by Maniatis, T., et al., *Molecular Cloning (A Laboratory Manual)*, Cold Spring Harbor Laboratory, second edition, 1989), and are well known in the art.

Libraries containing clones encoding a desired protein or a desired transcriptional regulatory element may be screened and a desired clone identified by any means which specifically selects for the DNA of interest. For example, if a clone to an acid α -amylase is desired, such a clone may be identified by any means used to identify acid α -amylase protein or mRNA, including, for example, a) by hybridization with

-16-

an appropriate nucleic acid probe(s) containing a sequence(s) specific for the DNA of this protein, or b) by hybridization-selected translational analysis in which native mRNA that hybridizes to the clone in question is translated *in vitro* and the translation products are further characterized, or, c) if the cloned genetic sequences are themselves capable of expressing mRNA, by immunoprecipitation of a translated protein product produced by the host containing the clone.

Oligonucleotide probes specific for a desired protein can be used to identify a desired clone. Such probes can be designed from knowledge of the amino acid sequence of the desired protein. The sequence of amino acid residues in a peptide is designated through the use of the commonly employed three-letter or single-letter designations. A listing of these three-letter and one-letter designations may be found in textbooks such as *Biochemistry*, Lehninger, A., Worth Publishers, New York, NY (1970). As used herein, when the amino acid sequence is listed horizontally, the amino terminus is intended to be on the left end and the carboxy terminus is intended to be at the right end.

Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid. The peptide fragments are analyzed to identify sequences of amino acids that may be encoded by oligonucleotides having the lowest degree of degeneracy. This is preferably accomplished by identifying sequences that contain amino acids that are encoded by only a single codon.

Although occasionally an amino acid sequence may be encoded by only a single oligonucleotide sequence, frequently the amino acid sequence may be encoded by any of a set of similar oligonucleotides. Importantly, whereas all of the members of this set contain oligonucleotide sequences that are capable of encoding the same peptide fragment and, thus, potentially contain the same oligonucleotide sequence as the gene which encodes the peptide fragment, only one member of the set contains the nucleotide sequence that is identical to the exon coding sequence of the gene. Because this member is present within the set, and is capable of hybridizing to DNA even in the presence of the other members of the set, it is possible to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the peptide.

Therefore, using the genetic code, one or more different oligonucleotides can be identified from the amino acid sequence, each of which would be capable of encoding the desired protein. The probability that a particular oligonucleotide will, in fact, constitute the actual protein's encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in the host cell. Such "codon usage rules" are disclosed, for example, by Lathe, R., et al., *J. Molec. Biol.* 183:1-12 (1985). Using the "codon usage rules" of Lathe, a single oligonucleotide sequence, or a set of oligonucleotide sequences, that contain a theoretical "most probable" nucleotide

-18-

sequence capable of encoding the acid α -amylase of the invention is identified.

In addition to the codon usage rules, oligonucleotide design can may utilize the use of deoxyinosine at ambiguous codon positions. This approach is particularly useful when the required DNAs sequence is derived from a poorly characterized organism like *B. acidocaldarius* (Takahaski et al., *Proc. Natl. Acad. Sci. USA* 82:1931-1935 (1985)).

The suitable oligonucleotide, or set of oligonucleotides, that are capable of encoding a fragment of the desired gene (or which is complementary to such an oligonucleotide, or set of oligonucleotides) may be synthesized by means well known in the art (see, for example, *Synthesis and Application of DNA and RNA*, S.A. Narang, ed., 1987, Academic Press, San Diego, CA) and employed as a probe to identify and isolate the cloned gene by techniques known in the art. Techniques of nucleic acid hybridization and clone identification are disclosed by Maniatis, T., et al. (In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1982)), and by Hames, B.D., et al., in: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985)), which references are herein incorporated by reference. Those members of the above-described gene library which are found to be capable of such hybridization are then analyzed to determine the extent and nature of the sequences which they contain.

To facilitate the detection of the desired encoding sequence, the above-described DNA probe may be labeled with a detectable group. Such detectable

group can be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of nucleic acid hybridization and in general most any label useful in such methods can be applied to the present invention. Particularly useful are radioactive labels, such as ^{32}P , ^3H , ^{14}C , ^{35}S , ^{125}I , or the like. Any radioactive label may be employed which provides for an adequate signal and has a sufficient half-life. The oligonucleotide may be radioactively labeled by means well-known in the art, for example, "nick-translation" and T4 DNA polymerase replacement synthesis.

Alternatively, polynucleotides are also useful as nucleic acid hybridization probes when labeled with a non-radioactive marker such as biotin, an enzyme or a fluorescent group.

Thus, in summary, the actual identification of acid α -amylase peptide sequences permits the identification of a theoretical "most probable" DNA sequence, or a set of such sequences, capable of encoding such acid α -amylase. By constructing an oligonucleotide complementary to this theoretical sequence (or by constructing a set of oligonucleotides complementary to the set of "most probable" oligonucleotides), one obtains a DNA molecule (or set of DNA molecules), capable of functioning as a probe(s) for the identification and isolation of clones directed to acid α -amylase.

The above discussed methods are, therefore, capable of identifying genetic sequences that are capable of encoding acid α -amylase, or fragments, variants and functional derivatives of acid α -amylase. In order to further characterize the cloned acid α -

-20-

amylase (or its fragment, variant or functional derivative) and especially, in order to produce recombinant acid α -amylase, it is desirable to express the proteins which the sequences encodes.

III. Expression of Proteins Using the Expression Vectors of the Invention

Expression of acid α -amylase protein allows the identification of clones that are capable of expressing the acid α -amylase protein of the invention, or fragments, variants or functional derivatives thereof. Characteristics unique to acid α -amylase that may be used to identify the acid α -amylase protein, fragment, variant or functional derivative include the ability to specifically bind acid α -amylase antibodies, the ability to elicit the production of acid α -amylase antibodies that are capable of binding to the native protein, and the ability to provide an enzymatic function specific to acid α -amylase, such as, for example, enzymatic stability at an acidic pH at which other "non-acidic" α -amylase enzymes are relatively unstable, among others.

In a preferred embodiment, amylase-negative bacterial strains are used as hosts. Especially preferred are amylase negative mutants of *Bacillus* or *L. plantarum* (which does not produce amylase by definition).

To express the acid α -amylase of the invention, transcriptional and translational signals recognizable by an appropriate host are necessary. C l o n e d

-21-

sequences encoding acid α -amylase, its fragments, variants or functional derivatives, obtained through the methods described above, and preferably in a double-stranded form, may be operably linked to sequences controlling transcriptional expression in an expression vector. Such sequences may be introduced into a host cell to produce recombinant acid α -amylase, its fragments, variants and functional derivatives.

In addition to transcriptional signals, it may also be advantageous to use translational and/or secretion signals derived from other bacterial sources provided that such translational and secretion signals are functional in the chosen host cell. This is discussed further *infra*.

According to the invention, any prokaryote host may be utilized. In a preferred embodiment, a member of the *Bacillus* or *Lactobacillus* genera are used as the host cell for expressing the desired protein of the invention. Such members include *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. polymyxa*, *B. stearothermophilus*, *B. theroproteolyticus*, *B. coagulans*, *B. thuringiensis*, *B. megaterium*, *B. cereus*, *B. natto*, and, *B. acidocaldarius*. In an especially highly preferred embodiment, the host cell is *Lactobacillus*.

Lactobacillus species that are associated with silage and are especially preferred as hosts in the processes of the invention are *L. plantarum*, *L. brevis*, *L. buchnerie*, *L. coryniformis*, *L. curvatus*, *L. casei*, *L. fermentum*, *L. acidophilus*, and *L. salivarius*.

-22-

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide like the acid α -amylase of the invention if the nucleic acid molecule contains expression control sequences that provide transcriptional regulatory information and such sequences are operably linked to the nucleotide sequence which encodes the acid α -amylase.

The precise nature of the regulatory regions needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribing and 5' non-translating (non-coding) sequences involved with initiation of transcription and translation respectively. Especially, such 5' non-transcribing control sequences will include a region which contains a promoter capable of functioning in the host cell.

Expression of a recombinant protein in prokaryotic hosts requires the use of regulatory regions functional in such hosts, and preferably prokaryotic regulatory systems. A wide variety of transcriptional and translational regulatory sequences can be employed, depending upon the nature of the prokaryotic host. Preferably, these regulatory signals are associated with a particular gene which is capable of a high level of expression in the host cell.

If desired, a fusion product of the desired protein may be constructed. For example, if the genetic sequence encoding a desired acid α -amylase (or its fragment, variant or functional derivative) does not possess a sequence encoding a signal sequence functional in a certain host, such signal sequence may be operably linked to the desired genetic sequence, thus allowing secretion of the protein from, or the

-23-

membrane compartmentalization of the protein in, the host cell. Such signal sequences may be designed with or without specific protease sites such that the signal peptide sequence is amenable to subsequent removal. Alternatively, the native signal sequence of a protein of the invention may be used.

In addition to using either a native signal sequence or a signal derived from some other bacteria, it is often useful to construct a signal sequence fusion sequence, utilizing only part of the native signal sequence. Potential fusion sites between two signal sequences are the hydrophobic regions or the helix breaker residue between the hydrophobic region and the C-terminal part of the signal sequence. Examples of useful fusion signal sequences are described in Applicants' copending application, U.S. Application No. 07/377,450, filed July 10, 1989 and incorporated herein fully by reference.

Transcriptional initiation regulatory signals that can be operably linked to the proteins of the invention can be selected which allow for repression or activation, so that expression of the operably linked genes can be modulated in a specific manner.

Where the native expression control sequences signals do not function satisfactorily in a desired host cell, then sequences functional in the host cell may be substituted.

Once the vector or DNA sequence containing the construct(s) is prepared for expression, the DNA construct(s) is introduced into an appropriate host cell by any of a variety of suitable means known in the art. In a preferred embodiment, *Lactobacillus* are

transformed essentially as described in von Wright et al., *Appl. Environ. Microbiol.* 56:2029-2035 (1990).

To transform a host cell with the DNA constructs of the invention many vector systems are available, depending upon whether it is desired to insert the genetic DNA construct into the host cell chromosomal DNA, or to allow it to exist in an extrachromosomal form. When it is desired to maintain a vector in an extrachromosomal form the vector should provide an origin of replication capable of functioning in the host.

Genetically stable transformants may also be constructed with vector systems, or transformation systems, whereby a desired protein's DNA is integrated into the host chromosome. Such integration may occur *de novo* within the cell or, in a most preferred embodiment, be assisted by transformation with a vector which functionally inserts itself into the host chromosome. For example, such vector may provide a DNA sequence element which promotes integration of DNA sequences in chromosomes. In a preferred embodiment, such DNA sequence element is a sequence homologous to a sequence present in the host chromosome such that the integration is targeted to the locus of the genomic sequence and targets integration at that locus in the host chromosome.

Cells that have stably integrated the introduced DNA into their chromosomes are selected by also introducing one or more markers which allow for selection of host cells that contain the expression vector in the chromosome, for example the marker may provide biocide resistance, e.g., resistance to antibiotics, or the like. The selectable marker gene

can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transformation.

A sequence may also be incorporated into a plasmid or other vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

In addition to antibiotic selection markers, one can (or should, if such antibiotic markers are undesirable in a specific process in which the hosts will be utilized) use natural selection markers based on, for example, nisin resistance, thymidine synthesis, lactose utilization or X-prolyl-dipeptidyl-aminopeptidase (*Appl. Environ. Microbiol.* 57:38-43 (1991)).

After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired protein. This expression can take place in a continuous manner in the transformed cells, or in a controlled manner.

The expressed acid α -amylase, or fragment, variant or functional derivative thereof may be isolated and purified in accordance with the method

-26-

described herein and using techniques known in the art. However, in a highly preferred embodiment, no isolation or purification of the product is needed as the host is directly inoculated into the process medium.

The genetic sequences, protein sequences, vectors and methods of the invention are useful in many applications of the food and beverage industry. Especially, the hosts of the invention are useful when directly inoculated into foodstuff. The lactic acid bacteria of the invention may be utilized for the preparation of fermented food and beverages as lactic acid bacteria play an essential role in the preparation of such fermented food and beverages. The *Lactobacilli* of the invention may also be applied in silage preparation and as probiotics in human and animal health. The hosts containing the sequences and methods taught herein, together with, if necessary, current knowledge of the genetics and biochemistry of lactic acid bacteria, especially of *Lactococci* and *Lactobacillus*, may be used for the construction of bacterial agricultural process strains that have been optimized for different agricultural and food preparation applications using the sequences and methods of the invention. The genetics of Lactic acid bacteria are known in the art and are reviewed, for example, in *Biochimie* 70:No. 3 and No. 4 (1988) and in *FEMS Microbiol. Rev.* 87:No. 1 and No. 2 (1990).

For example, one optimization using the sequences and methods of the invention is the addition of heterologous acid α -amylase activities, and especially hosts expressing such activities, to any process strain. Highly desirable hosts that may be utilized

-27-

as process strain host microorganisms include *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. polymyxa*, *B. stearothermophilus*, *B. thermoproteolyticus*, *B. coagulans*, *B. thuringiensis*, *B. megaterium*, *B. cereus*, *B. natto*, and, *B. acidocaldarius*.

Especially the acid α -amylase activity of the invention would be valuable for these strains as lactic acid bacteria are widely used for preservation of many starch containing raw materials (e.g., cereal grains, edible roots and crop residues).

Lactobacillus carrying and expressing the acid α -amylase gene of the invention, or a fragment, variant or functional derivative thereof, would be also extremely useful in food and fodder preparation. Such expression may occur under the control of the homologous (native) regulatory regions or under the control of heterologous promoter and/or signal sequences, especially those of *Lactococcus* or *Lactobacillus* origin.

Additionally, the *Lactobacillus* hosts of the invention, carrying the acid amylase of the invention are useful for the preservation of feed and fodder material rich in starch, e.g., cereal grains (siliaged with crimping), corn (maize), maize cobs and alfalfa.

Another specific application of these strains is their use in starter feeds for calves, in milk replacers for calves younger than four weeks (to increase digestibility of starch and for probiotic action) and in starter feed for piglets.

Furthermore, these strains can be effectively used for preservation of material containing cereal and slaughter scraps for fodder use.

-28-

The examples below are for illustrative purposes only and are not deemed to limit the scope of the invention.

EXAMPLES

Example 1

Isolation of the Acid α -Amylase Enzyme from *Bacillus acidocaldarius*

Bacillus acidocaldarius (B. ac.) strain ATCC 27009 was grown on agar plates containing (per liter): 1 g yeast extract, 0.2 g $(\text{NH}_4)_2\text{SO}_4$, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.6 g KH_2PO_4 and 2.5 g maltose, pH 4.5. To isolate the α -amylase enzyme, the entire surface of the agar plate was inoculated with B. ac. cells and the plates were incubated 3-5 days at 55°C. The cultures were then suspended in 0.5 M sodium acetate, pH 5.0, and the cells were removed by centrifugation at 8000 g for 20 min. The supernatant was centrifuged again at 40,000 g for 30 min and proteins in 50 ml of the cleared growth medium were precipitated at 0°C by slow addition of $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 70% (w/v). After allowing the precipitate of the proteins to form for 30 min at 0°C, the precipitated proteins were collected by centrifugation at 10,000 g for 20 min. The pellet was dissolved in 3 ml of 20 mM BisTris, pH 5.8, and applied onto a Bio-Gel P-200 (Sigma, Richmond, USA) column (1.5 x 45 cm). The elution was carried out in the same buffer. Rapid screening for amylase activity in the eluted fraction was performed using plate assay. The assay plates contained 1.5% agar, 0.2% starch, 20 mM CaCl_2 , 50 mM sodium acetate, pH 5.0.

Samples were applied into wells made in agar and, after incubation for 2-10 hours at 55°C, the enzyme activity was detected by spreading 0.01 M I₂/0.01M KI solution onto the plates. The enzyme activity can be detected as a halo around the agar well. For quantitative assay of the acid α -amylase activity, the Phadebas® amylase test (Pharmacia) was used.

The amylase containing fractions from Bio-Gel P-200 were pooled and concentrated by ultrafiltration in a Novacell™-Omegacell™ apparatus (Filtron, Northborough, Massachusetts, USA). The concentrate was rechromatographed in a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden) in 20 mM Bis-Tris, pH 5.8. The amylase containing fractions were concentrated as above by ultrafiltration and stored at -20°C.

To detect the amylase activity in SDS-polyacrylamide gels, zymography was used (Harris, H. et al., *Handbook of Enzyme Electrophoresis in Human Genetics*, North-Holland, Amsterdam, and, *Methods in Enzymology* 22:578 (1971)). After SDS electrophoresis, the gel was washed for 15 min in 50 mM sodium acetate, pH 5.0 and put onto an amylase assay plate. After incubation at 55°C for 10 hours the enzyme activity was detected as above. When the amylase(s) produced by *B. ac.* strain ATCC 27009, isolated and partially purified as described above, were analyzed by SDS-PAGE and zymography, two forms with relative molecular weights of 90,000 and 160,000 were detected (Figure 1). Similar results were obtained when the SDS-PAGE preceding zymography was performed under nonreducing conditions. This suggests that the 160 kd form was not a disulfide linked dimer of the 90 kd form. Under

-30-

the conditions in zymography the 160 kd form had apparently much higher specific activity (Figure 1).

To assay the amylase activity after SDS-PAGE by zymography, the SDS-gel was first incubated in 20 mM CaCl_2 , 50 mM sodium acetate, pH 5.0 for 15 min at room temperature. After this incubation, the SDS-gel was placed on an agarose gel (3mm thick, 1.0% agar, 0.2% starch, 20 mM CaCl_2 , 50 mM sodium acetate, pH 5.0). The gels were then incubated at 55°C for 10 hours. To reveal the amylase activity, the agarose gel was stained by spreading 0.01M I_2 /0.01M KI solution onto the gel. A clear halo in the dark background indicated the amylase activity.

In chromatofocusing (Huchthens, T.W. in: *Protein Purification*, J.C. Janson et al., eds., V.H.C. Publishers Inc., New York (1989)) and isoelectric focusing (der Lan et al., in: *Gel Electrophoresis of Proteins*, B.D. Hames et al., eds., IRL Press, Oxford) followed by zymography both the 160 kd and 90 kd polypeptides migrated as a broad peak and band, respectively, at pI 4.8. When the pH optimum of the enzyme(s) was measured, a symmetric curve with a relatively sharp pH optimum at pH 5 was obtained. The enzyme activity was about four fold at 60°C to that at 37°C (Figure 2). The above data suggests that the 90 kd polypeptide might be a degradation product of the 160 kd protein.

-31-

Example 2N-Terminus Sequencing of the Acid α -Amylase Gene

To sequence the acid α -amylase enzyme, SDS-PAGE was performed to separate the 90 kd and 160 kd polypeptides from the partially purified culture supernatant. After SDS-PAGE the gel was treated with 1 M KCl to visualize protein bands and the band corresponding the enzyme activity was excised. From the gel the protein was electroeluted using an ISCO model 1750 electrophoretic concentrator as described in *Advanced Methods in Protein Microsequence Analysis*, Wittmann-Liebold, B., Salnikow, J. and Erdman, V.A., eds., pp. 194-206, 1986, Springer-Verlag Berlin Heidelberg. Only the 90 kd polypeptide could be purified in an amount required for NH_2 -terminal sequencing. For NH_2 -terminal amino acid sequence analysis acid amylase was degraded in a gas/pulsed liquid sequencer (*J. Prot. Chem.* 7:242-273 (1988)) after electrophoretic transfer onto a polyvinylidene difluoride membrane Speicher, D.W. in: *Techniques in Protein Chemistry*, T.E.Hugli, ed., Academic Press., San Diego, CA (1989). When the 90 kd protein electrotransferred onto a polyvinyl difluoride membrane was degraded, a single amino acid sequence of NH_2 -Asp-Ile-Asn-Asp-Tyr was obtained.

For the cloning of the corresponding acid α -amylase gene, eight lysylendopeptidase-cleaved peptides of the 90 kd protein were purified as follows: After electroelution of the 90 kd protein from the gel, the eluate was freeze dried and the solid material was redissolved in 50 μl of 50 mM Tris/HCl, pH 9.0. 150 ng of lysylendopeptidase (Wako,

-32-

Dallas, USA) was added and the mixture was incubated at 30°C for 18 hours. The resulting peptides were separated by reverse phase chromatography on a Vydack 218 TPB5 (0.46 x 15 cm) column connected to a Varian 5000 liquid chromatograph. The peptides were eluted using a linear gradient of acetonitrile (0-60% in 90 min) in 0.1% trifluoroacetic acid. The peptides were sequenced after application on polybrene (2mg) pretreated glass fiber filters. The N-terminal sequence of the eight purified peptides derived from 90 kd protein are shown in Table 1. Three of the eight N-terminal sequences (Table 1 no. 2, 3 and 4) were used to synthesize oligonucleotides. The sequences of these oligonucleotides, used for screening of the *B. ac.*-gene library, are shown in Table 2.

TABLE 1

N-terminal sequences of the lysylendopeptidase cleaved peptides of the 90 kd protein.

Peptide	Sequence
1	tyr-asp-thr-ala-asp-tyr-phe-lys [SEQ ID No. 6]
2	leu-val-ser-asp-phe-gln-phe-ser-phe-pro-x-asp-pro-thr-ile-phe-tyr [SEQ ID No. 7]
3	ile-asp-pro-gly-phe-gly-thr-gln-gln-asp-x-leu-asn-leu-val-gln-ala [SEQ ID No. 8]
4	his-ala-val-ile-tyr-glu-ile-met-pro-asp-pro-phe-tyr-asn-gly-asn- ile-ala [SEQ ID No. 9]
5	leu-asp-tyr-leu-lys [SEQ ID No. 10]
6	ser-leu-gly-val-asn-thr-leu-tyr-leu-met-pro-val-phe-glu-ala [SEQ ID No. 11]
7	phe-gly-asn-phe-his-ser-asn-gly [SEQ ID No. 12]
8	gly-ile-tyr-val-gly-ala [SEQ ID No. 13]

-33-

Table 2

Oligonucleotides used for screening of the B.ac. - α -amylase gene. Oligos 370, 371 and 372 correspond to peptides 2, 3 and 4, respectively. "I" is inosine.

Oligonucleotide	Sequence
370	5' TA GAA GAT IGT IGG GTC III IGG GAA IGA GAA-CTG GAA GTC IGA IAC 3' [SEQ ID No. 3]
371	5' CA GTC CTG CTG IGT ICC GAA ICC IGG GTC GAT 3' [SEQ ID No. 4]
372	5' GC IAT GTT ICC GTT ITA GAA IGG GTC IGG CAT IAT CTC ITA IAT IAC IGC GTG 3' [SEQ ID No. 5]

Example 3

Cloning of the *B. acidocaldarius* Acid α -Amylase Gene

ATCC 27009 *B. ac.* cells were grown in a liquid medium containing [per liter] 1g yeast extract, 0.2g $(\text{NH}_4)_2\text{SO}_4$, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g CaCl_2 , 0.6g KH_2PO_4 and 1g glucose, pH 4.5 at 55°C until late logarithmic phase. The cells were collected and the chromosomal DNA was isolated according to Marmur, *J. Mol. Biol.* 3: 208-218 (1986)), except that the DNA was dissolved in saline after first ethanol precipitation. The chromosomal DNA was partially digested with *Hae*III and after agarose gel electrophoresis a fragment population of 4-6 kb was isolated. The DNA fragments were cloned in *E. coli* using λ -gt10 as the vector (Amersham) according to manufacturers recommendation. The *B. ac.* gene bank in *E. coli* was screened by plaque hybridization using oligonucleotides 370 [SEQ ID No. 3]; 371 [SEQ ID No. 4] and 372 [SEQ ID No. 5] (see table 2) as

radioactive probes. After hybridization a positive clone, with an 4.2 kb insert, was found. The insert was subcloned in M13 vectors and pBR322 and sequenced either by M13 method or using direct plasmid sequencing method.

The DNA sequence revealed an open reading frame of 3532 nucleotides starting from the 5' end of the 4.2 kb insert (see Figure 3 and [SEQ ID. No. 1]). Within the open reading frame, starting from the codon 342, a deduced amino acid sequence DINDY (see Figure 3) can be found. This sequence corresponds to the N-terminal amino acid sequence of the 90 kd protein. The open reading frame downstream of codon 342 codes for a 92 kd protein which is in good agreement with the purified M_r 90 kd band. Codon 342 is preceded by an open reading frame at the 5' end of the 4.2 kb clone, and there are no sequences resembling either promoter or SD-regions in the near vicinity of the N-terminal codon (codon 342). This strongly suggests that the 90 kd protein indeed is a breakdown product of a larger polypeptide and the regulatory regions of this polypeptide are outside of the 4.2 kb insert. Therefore, the *B.ac.* gene bank was rescreened using a 5' fragment of the 4.2 kb clone as a probe. This resulted in a new 5.1 kb clone which partially overlapped 5' end of 4.2 kb fragment. The organization of these two clones was confirmed by Southern blotting from the *B.ac.*-genome and the joint region was sequenced also directly from a relevant chromosomal PCR-fragment. The 5.1 kb clone was sequenced by M13 method.

The DNA sequence upstream of the 4.2 kb fragment revealed that open reading frame of the 4.2 kb fragment continued additional 400 codons. The open reading was preceded by a typical promoter - SD-region, and the deduced N-terminal amino acid sequence showed typical features of the bacterid signal peptide (see Figure 3). The combined

-35-

open reading frame codes for a protein of 150 kd which is in good agreement with the 160 kd band shown in the SDS-PAGE - zymography. The deduced amino acid sequence revealed sequences that are typical to other amylolytic enzymes and surprisingly, a C-terminus that resembles a hydrophobic anchor sequence.

To combine the two parts of the amylase gene the relevant DNA fragments of the 5.1 kb and 4.2 kb clones have been ligated in to a pHP13 plasmid vector (*Mol. Gen. Genetics* 209:335-342 (1987)) and transformed in *Bacillus subtilis*. The DNA analysis of the acid amylase/pHP13 hybrid plasmid confirmed the intact acid amylase gene was stably maintained in *B. subtilis*. This hybrid plasmid was designated pKTH2034.

Example 4

Expression of the Acid α -Amylase Gene in *Lactobacillus*

Using the clone described herein, the *B.ac.* α -amylase gene is expressed in *Lactobacillus* host cells. The method is described in detail in Examples V, VI, VII and X, of the Applicants' copending Application Serial No. 377,450, filed July 10, 1989, the specification of which is incorporated herein by reference in its entirety as if set forth in full.

Briefly, plasmids as exemplified by pKTH 1797, pKTH 1798, pKTH 1799, pKTH 1801, pKTH 1805, pKTH 1806, pKTH 1807 and pKTH 1809 contain promoter and secretion promoting signals. The promoter and secretion promoting sequences of these plasmids can direct the expression of heterologous gene and secretion of gene product in Gram positive host cells such as *Bacillus*, *Lactobacillus* and *Lactococcus*.

To obtain expression of the acid α -amylase gene and secretion or export of the gene product, a host cell such as *Lactobacillus* is transformed with a plasmid comprising

-36-

the promoter and secretion promoting sequences described above and the gene coding for the mature acid amylase protein described herein. In addition of using a plasmid that is able to replicate in *Lactobacillus* host, a nonreplicative plasmid (e.g., pBR322 or pE194), bearing random fragments of *Lactobacillus* chromosome, can be used. Transformation with nonreplicative plasmid results in integration of the acid amylase gene in the *Lactobacillus* chromosome.

The host containing the amylase gene is cultured in a suitable medium allowing expression of the protein and the protein is recovered from the cell wall layer or culture medium or the host cell, producing the α -amylase activity, is directly used in the required process. The *Lactobacillus* host of the invention, modified to express the acid= α -amylase gene, may be utilized for the processing of food and feed as in the following examples.

Example 5
Processing of Forage

Forage, for instance grass, is harvested by either a frail harvester or a precision chopper attached to a wagon of a tractor. Preservation solution is added to the chopped forage mass during the harvesting in the chopper/harvester using equipment designed for this purpose. Th preservation solution is prepared by adding an inoculant (a *Lactobacillus* strain as described above, capable of producing the acid amylase of the invention), to a bottle or tank which contains half of its volume as lukewarm water. This water should not be so hot as to kill the bacteria. After shaking to evenly distribute the inoculant, the rest of the volume of the bottle is filled

-37-

by lukewarm water. Usually farmers use bottles of 30 liters or tanks of 200 liters. The inoculant can be either in liquid form or as a dried powder of the acid amylase-expressing hosts of the invention. The inoculant may be prepared in fermentors using whey-based medium and the culture is grown to a cell density of approximately 10^9 cells/ml. After the culture has reached this density, the cells are collected by centrifugation and lyophilized to a final density of 10^{11} - 10^{12} cells/g of dry weight. Twenty grams of this lyophilized material or equal amount of cells in a liquid concentrate is mixed with 30 liters of water as described above. Five liters of this diluted solution is applied per ton of forage.

Alternatively, the prepared preservation solution can also be added to the forage mass after the wagon has been emptied in the silo in which fodder is ensiled.

Example 6
Animal Feed Additive

A concentrate mixture (powdered, granulated or pelleted) is manufactured using standard manufacturing systems. Each component in the mixture is separately weighed and then mixed together before pelleting, for instance. An inoculant (as described in Example 5) can be added or mixed into the mixture as one of the components. The concentrate, including the inoculant, can be fed to domestic animals, such as, for example, cows, calves, pigs, piglets, etc.

The inoculant can also be fed separately as a specially formulated powder, tablet or a liquid product. The inoculant is generally included in a carrier ingredient, for instance, skim milk powder. These products can be fed to domestic animals directly, as as a component together with the other feeds or in the drinking water of the animal.

For each purpose, an inoculant is manufactured so that it contains 10^{11} - 10^{12} cfu/g dry inoculant powder. The inoculant may be used in any concentration which is necessary to achieve the desired effect. Especially, when used as an additive to the animals' feed, the inoculant is used at 0.01-0.02% in the concentrate mixture.

The advantage of feeding the inoculant of the invention to domesticated farm animals such as the above is that by letting the hosts of the invention colonize the intestinal tract of the animal, the animal is better able to digest the silage or fodder that it is fed, as the acid stable amylase secreted by such hosts would better degrade the ingested foodstuff.

All references cited herein are incorporated herein by reference. While this invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various

-39-

changes and modifications could be made therein without departing from the spirit and scope thereof.

-40-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Teija Koivula,
Mervi Sibakov, and
Ilkka Palva
- (ii) TITLE OF INVENTION: Acid α -Amylase
- (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Sterne, Kessler, Goldstein & Fox
 - (B) STREET: 1225 Connecticut Avenue, N.W.,
Suite 300
 - (C) CITY: Washington
 - (D) STATE: District of Columbia
 - (E) COUNTRY: United States of America
 - (F) ZIP: 20036
- (v) COMPUTER READABLE FORM;
 - (A) MEDIUM TYPE: Floppy Disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: Ascii
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Michele Ann Cimbala
 - (B) REGISTRATION NUMBER: 33,851
 - (C) REFERENCE/DOCKET NUMBER: 1155.0090000
- (viii) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (202) 833-7533

(2) INFORMATION FOR SEQ ID NO :1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 4140 bases
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Double Stranded
 - (D) TOPOLOGY: Linear
- (ii) MOLECULAR TYPE: Nucleic Acid
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATGTGGGTC GCCTCCTCTG CGATGGGACA TCTCACTTAC ATTTTAAACAG TGAAGGGTG	60
GGGAAGGGAA CAAGGGTCTT GAGAGAAAAG AAAGGATTCG GACAATGAAC GATGCAAGAG	120
CACCATTGAC GTCGGGAAGG CTGAGCGTAT AATCCAGGAC GACAACGTTA GTGCAATCGT	180
TTGCACAAGA GGAGGTTGAT GCACGTGAAG TGGACGGGTG CCGCGAGCCT TGCAGACACC	240

-41-

ATCGTCATGT CTGCAGCGCT TCCGTTCCGG GCGTTTGAAA ACACCCGTTT CCTCGGCACG	300
GAACGCGTTC TGGCGGCGAC CGCGGCGGAT AGTACTAACG CCTCGCCCCC GACCGGGGCG	360
TCCGCAGGCG GTGGCACAGC AGGAGAGACC TACTCGAATG CCGTGCAGAT TGTGAGCGGC	420
AATGGCTTTC GCGCTGGAAA CGGGGCCACG GTCGTCTCG CTGTGAATGG ACTGAACTTG	480
AACCCCTCGA GTTTCAAGGT TGTGGTATCG AATAGCCTCA ACGGCGTGAT CGACGTCACA	540
AGTGATTCCG TGCTAACGGG GCGAATTG ATTGCCTTGC ACCTTCCTGC CGGGGATGAC	600
GGGCTTGGCG CCGGTACGTA CACGGTCACC GTCGAAAGTG GAGGTGATTC GGTCTCGACG	660
CCTTCAGGAC AGGGACTTCA GGTTCATGCCT TATACGACGG CCGACACCAT TCAGTGGGAT	720
GGAATCTACA CATCCGATGG CCGATGTAC GTGTCAGATC CGAATCCCTC CCCTGGACAA	780
GAGGTGACGA TCAGCCTTCG AGCGTACAGC GGAACCTGA CGAAGGTGAT CCTGAATTGC	840
TGGGACACCG CACAAAACAA GGGTTTCCAG GTCGAGATGT CTCCAGGCAG AACGTTTGGA	900
CCGTATCAGC TCTGGTCTGC CACGATCCCC GCTTCAAACG GCGGAACGAT CTATTATCGC	960
TTTGACATAT ACGATGGCAC CAGTTTTCG TGTCTCTCAG GTGACGGA CTGACACGTCT	1020
GACGACATCA ACAACAATTT CCCGTTGCCC GTGGGGACGG TCACGCTTTC GACACTTCAG	1080
GCGAATCCCG GTGATACGGT GACGCTCTCC GACCCTGTAG GTGACTTCGC CGGAAGCCAG	1140
GATCAACCCA ATCACACGGT GATACGGTTT GTCAACTCGT CGGGCGAAAC GGCGGCCACA	1200
GTCAATGGGA CGAACGCGAG CTGGAACAGC GTGCAGTTCA CAGTCCCACA GAGCCTTCCA	1260
AACGGCTTGT ATCGCGTCGA GATCGACAGC GTCGCCAAGG ACGCGGATGG GGTGGTCAAT	1320
GTCGAATTGG ACAGGAGTGC GGAGCTTATT GTAGGGCCTC TGCCCGCGTG GATGCAAGCG	1380
TATGCACATG ATTCGTTTCA GCGTTCTAC CGATCGCCTT TCGGAGCCGT GTCCACAGGA	1440
ACCCCCATCA CGCTTCGCCT GCGGGCTCCG CTCAGCGTGA AGAGTGCAGC GCTTCGCCTC	1500
TGGGGGGCAG CGGATCAGTC AGGCGAGATC GACCTGCCGA TGCAGAAGCT CCAAATGTCTG	1560
GGAGACGAGT TGGCGCAACA AACCGGCGTG CAGGACATCA ACGACTACAC GTGGTGGACG	1620
GTGACCATCC CTGCGGCGGA TGTGACCACA CCCGGGACGA TGTGGTATCA GTTCGTGACG	1680
GAGACGGACA CTGGCCAGGT GGTCTACTAC GATGACAATG GAGCTCAGCT TGAAGGGCCT	1740
GGCCAGGTG GGTGTCTTC CGACGGACCG AGCTACCAGA TCAGCGTATA CGAACGGGGA	1800
TTTCAGACGC CAGATTGGCT GAAGCAGCC GTGATCTACG AAATCATGCC GGATCGGTTT	1860
TACAATGGCA ATATCGCCAC GGAGGAGAAT CGAATACGC AAAAGGGGAT TTATGTAGGG	1920
GCCGATGGAA CGGAGTCATT AGGCCCCATC CAGTTCCAG AGAACTGGGA CTCGCGCCCC	1980
TATGATCCGA ATATTCCTCC GTTATCTGAT CCCAAAATTG CCAGTCTCG AGGCAATGGC	2040

-42-

CAATGGAACA TTGACTTTTT CGGAGGTGAT TTGCAGGGCA TCGAGGATAA GCTGGACTAC	2100
CTGAAGAGCC TTGGAGTCAA TACGCTGTAT CTGATGCCCC TCTTTGAGGC GGAATCCAAT	2160
CACAAATATG ACACAGCCGA CTATTTCAAG ATTGACCCTG GATTTGGAAC GCAGCAGGAC	2220
TGGCTGAATC TCGTACAGGC TGCACACGCG AAGGGGTTC ATATCATTCT CGACGGGGTG	2280
TTCAAGATA CCGGAAGTGA CAGCGTATAT TTCAACAAGT TCGGGAACCT CCACTCCAAC	2340
GGTGCCTGGC AGGCGTACCT GAAGAACCAG CCGTCGCTGT CGCCCTACTA CTCGTGGTAC	2400
GTGTGGACAG GGAACACCTC AAACCCATAC GATTTCGTGT TTCAGATCGA CACGCTGCCA	2460
CTTACGGACA CGTCGAACCC CGCCTATCAG CGATTCTGT ATGGGAGCGA CAACTCAGTC	2520
GCGCGTGTGT GGATCCGGGA AGGTGCGGAC GGATGGCGCT TGGACTCGGC CGACAACGGG	2580
AATTTCAACA CGGCATGGTG GGGTGGCTTT CGGCAGGCCG TGAAATCGAT CGATCCCAAC	2640
GCAGCGATCA TCGGCGAGAT CTGGGACAAT GCGACGAATG ACAATGGAAC GGATTGGTTG	2700
ACGGGATCGA CCTTCGACAG TGTAATGAAC TACCAGTTC GGAACGCCGT GATCGACTTC	2760
TTCCCGGCA CGTACAACGA CGGAAACGTG CAGCACCACG CCGTCGACGC TCGGGATTTC	2820
AACCAGGAAC TGATGCGCCT GTACAGTGAA TATCCTCTGC AGTCGTTCTA CTCGATGATG	2880
AACCTTGTCG ATTCGCAAGA CACCATGCGG ATCCTGACCA TCTTGAGAA CGCGCCGCAG	2940
CCAGGCGATC TATCCGCGCT CCAGCAGGAT GAGTACAGGC CGTCTCCTGC GGCTGAACAG	3000
TTGGGGATCG AGAGGCTGAA GCTTGATATG GACTTTCAAT TCAGCTTCCC GGGCGATCCG	3060
ACCATCTTCT ACGGCGACGA GGCAGGGCTC ACTGGTTATT CGGATCCCCT CAATCGTCGG	3120
ACCTATCCGT GGGACAACCA GAATCTCGAT CTCCTGAACC ACTACGCAA GCTCGGGGCC	3180
ATTCGAAACG CCAATCCTGT GCTTCAGACG GGGGATTTC CGCCGTTGTA CGCACAGGGC	3240
ATGGTGATCG CATTGCAAG GACCATTCCG AATGGGCGAG ATGTCTTCGG TGTGCCAGCG	3300
GAGGATGCCA CGGCCATTGT GGCATCAAC AATCAGAAC AAGCTATCAC CGTGACCATT	3360
CCGACGGATG GGACGGTTGC GGACGGGTCC ACGATGCTCG ATGAACTGAA CAACCACTGG	3420
TACAAGGTGC AGAATGGTGG CATTACACTC ACGCTGCAAT CGTATCAAGG TGCCATTTTG	3480
GTGACGCCGA GCGACGCGCC GATGGCTTAT CTGCAAGAGG AGGATTCTCA GAACGAGATT	3540
GCGTGGACGC CTGTGCAAGG TGCCATCGGT TATCGCGTCT GGAGACAGAA TCCGAATGGA	3600
CAATGGGTGC CCTTTGGACC TGTGCTTCCT GCCACGACT TGAGTGTAC GGTGGAACGC	3660
GATGCATATG CGCAAACGTT TGCTGTACAA GCGCTGTTTT CGGCGTCTGA TCACGCCCAG	3720
TCTCCGGTGT CGGCACCTAA GACGGTATCG CTTCCCGTCG ATGTGCCCGC GGTACGCCCTG	3780
AGTCAGCCGA TCGTTAGTGG TCGTGTGGTT GGAGATCGTG CGATGGTCTC GATCACGCCG	3840

-43-

GTTCAGGCG CGACGCAGTA TGTGATCTAC CAGAGACAGG GCGACGGATC GTATGCTCCG	3900
GTCGCGACGG TCTCCACAAG TGGCGATTCC GCAGCTATAG GGAAGTTCC TGCGCAAGGT	3960
CCGGCCAACT CGCCTCACGC GACGATTCGC GTGACAGTGC CCGTACCTGC AGGTTTCTCG	4020
TCGGTGACCT ACCGCGTGGC TGCGCAAAAC GAAGATGGGC AAGCTGTGAC CAATCCATTG	4080
ACCCTATCGC TCTCGAAAAA GTGATGCCTC GCGAAAAGGG CTATCGGAAT TTTTTCGAGA	4140

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1341 amino acids
- (B) TYPE: Amino Acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: Linear

(ii) MOLECULAR TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	His	Val	Lys	Trp	Thr	Gly	Ala	Ala	Ser	Leu	Ala	Thr	Thr	Ile	Val	1	5	10	15
Met	Ser	Ala	Ala	Leu	Pro	Phe	Ala	Ala	Phe	Glu	Asn	Thr	Arg	Ser	Val	20	25	30	
Gly	Thr	Glu	Arg	Val	Leu	Ala	Ala	Thr	Ala	Ala	Asp	Ser	Thr	Asn	Ala	35	40	45	
Ser	Pro	Pro	Thr	Gly	Ala	Ser	Ala	Gly	Gly	Gly	Thr	Ala	Gly	Glu	Thr	50	55	60	
Tyr	Ser	Asn	Ala	Val	Gln	Ile	Val	Ser	Gly	Asn	Gly	Phe	Arg	Ala	Gly	65	70	75	80
Asn	Gly	Ala	Thr	Val	Val	Leu	Ala	Val	Asn	Gly	Leu	Asn	Leu	Asn	Pro	85	90	95	
Ser	Ser	Phe	Lys	Val	Val	Val	Ser	Asn	Ser	Leu	Asn	Gly	Val	Ile	Asp	100	105	110	
Val	Thr	Ser	Asp	Ser	Val	Leu	Thr	Gly	Pro	Asn	Ser	Ile	Ala	Leu	His	115	120	125	
Leu	Pro	Ala	Gly	Asp	Asp	Gly	Leu	Gly	Ala	Gly	Thr	Tyr	Thr	Val	Thr	130	135	140	
Val	Glu	Ser	Gly	Gly	Asp	Ser	Val	Ser	Thr	Pro	Ser	Gly	Gln	Gly	Leu	145	150	155	160
Gln	Val	Met	Pro	Tyr	Thr	Thr	Ala	Asp	Thr	Ile	Gln	Trp	Asp	Gly	Ile	165	170	175	

-44-

Tyr Thr Ser Asp Gly Ala Met Tyr Val Ser Asp Pro Asn Pro Ser Pro
 180 185 190
 Gly Gln Glu Val Thr Ile Ser Leu Arg Ala Tyr Ser Gly Asn Leu Thr
 195 200 205
 Lys Val Ile Leu Asn Cys Trp Asp Thr Ala Gln Asn Lys Gly Phe Gln
 210 215 220
 Val Glu Met Ser Pro Gly Arg Thr Phe Gly Pro Tyr Gln Leu Trp Ser
 225 230 235 240
 Ala Thr Ile Pro Ala Ser Asn Gly Gly Thr Ile Tyr Tyr Arg Phe Asp
 245 250 255
 Ile Tyr Asp Gly Thr Ser Phe Ala Cys Leu Ser Gly Asp Gly Leu His
 260 265 270
 Thr Ser Asp Asp Ile Asn Asn Asn Phe Pro Leu Pro Val Gly Thr Val
 275 280 285
 Thr Leu Ser Thr Leu Gln Ala Asn Pro Gly Asp Thr Val Thr Val Ser
 290 295 300
 Asp Pro Val Gly Asp Phe Ala Gly Ser Gln Asp Gln Pro Asn His Thr
 305 310 315 320
 Val Ile Arg Phe Val Asn Ser Ser Gly Glu Thr Ala Ala Thr Val Asn
 325 330 335
 Gly Thr Asn Ala Ser Trp Asn Ser Val Gln Phe Thr Val Pro Gln Ser
 340 345 350
 Leu Pro Asn Gly Leu Tyr Arg Val Glu Ile Asp Thr Val Ala Lys Asp
 355 360 365
 Ala Asp Gly Val Val Asn Val Glu Leu Asp Arg Ser Ala Glu Leu Ile
 370 375 380
 Val Gly Pro Leu Pro Ala Trp Met Gln Ala Tyr Ala His Asp Ser Phe
 385 390 395 400
 Gln Ala Phe Tyr Arg Ser Pro Phe Gly Ala Val Ser Thr Gly Thr Pro
 405 410 415
 Ile Thr Leu Arg Leu Arg Ala Pro Leu Ser Val Lys Ser Ala Thr Leu
 420 425 430
 Arg Leu Trp Gly Ala Ala Asp Gln Ser Gly Glu Ile Asp Leu Pro Met
 435 440 445
 Gln Lys Leu Gln Met Ser Gly Asp Glu Leu Ala Gln Gln Thr Gly Val
 450 455 460
 Gln Asp Ile Asn Asp Tyr Thr Trp Trp Thr Val Thr Ile Pro Ala Ala
 465 470 475 480
 Asp Val Thr Thr Pro Gly Thr Met Trp Tyr Gln Phe Val Thr Glu Thr
 485 490 495

-45-

Asp Thr Gly Gln Val Val Tyr Tyr Asp Asp Asn Gly Ala Gln Leu Glu
 500 505 510
 Gly Pro Gly Gln Val Gly Leu Ser Ser Asp Gly Pro Ser Tyr Gln Ile
 515 520 525
 Ser Val Tyr Glu Arg Gly Phe Gln Thr Pro Asp Trp Leu Lys His Ala
 530 535 540
 Val Ile Tyr Glu Ile Met Pro Asp Arg Phe Tyr Asn Gly Asn Ile Ala
 545 550 555 560
 Thr Glu Glu Asn Pro Asn Thr Gln Lys Gly Ile Tyr Val Gly Ala Asp
 565 570 575
 Gly Thr Glu Ser Leu Gly Pro Ile Gln Phe His Glu Asn Trp Asp Ser
 580 585 590
 Pro Pro Tyr Asp Pro Asn Ile Pro Pro Leu Ser Asp Pro Lys Ile Ala
 595 600 605
 Ser Leu Arg Gly Asn Gly Gln Trp Asn Ile Asp Phe Phe Gly Gly Asp
 610 615 620
 Leu Gln Gly Ile Glu Asp Lys Leu Asp Tyr Leu Lys Ser Leu Gly Val
 625 630 635 640
 Asn Thr Leu Tyr Leu Met Pro Val Phe Glu Ala Glu Ser Asn His Lys
 645 650 655
 Tyr Asp Thr Ala Asp Tyr Phe Lys Ile Asp Pro Gly Phe Gly Thr Gln
 660 665 670
 Gln Asp Trp Leu Asn Leu Val Gln Ala Ala His Ala Lys Gly Phe His
 675 680 685
 Ile Ile Leu Asp Gly Val Phe Glu Asp Thr Gly Ser Asp Ser Val Tyr
 690 695 700
 Phe Asn Lys Phe Gly Asn Phe His Ser Asn Gly Ala Trp Gln Ala Tyr
 705 710 715 720
 Leu Lys Asn Gln Pro Ser Leu Ser Pro Tyr Tyr Ser Trp Tyr Val Trp
 725 730 735
 Thr Gly Asn Thr Ser Asn Pro Tyr Asp Ser Trp Phe Gln Ile Asp Thr
 740 745 750
 Leu Pro Leu Thr Asp Thr Ser Asn Pro Ala Tyr Gln Arg Phe Val Tyr
 755 760 765
 Gly Ser Asp Asn Ser Val Ala Arg Val Trp Ile Arg Glu Gly Ala Asp
 770 775 780
 Gly Trp Arg Leu Asp Ser Ala Asp Asn Gly Asn Phe Asn Thr Ala Trp
 785 790 795 800
 Trp Gly Gly Phe Arg Gln Ala Val Lys Ser Ile Asp Pro Asn Ala Ala
 805 810 815

-46-

Ile Ile Gly Glu Ile Trp Asp Asn Ala Thr Asn Asp Asn Gly Thr Asp
 820 825 830
 Trp Leu Thr Gly Ser Thr Phe Asp Ser Val Met Asn Tyr Gln Phe Arg
 835 840 845
 Asn Ala Val Ile Asp Phe Phe Arg Gly Thr Tyr Asn Asp Gly Asn Val
 850 855 860
 Gln His His Ala Val Asp Ala Ala Gly Phe Asn Gln Glu Leu Met Arg
 865 870 875 880
 Leu Tyr Ser Glu Tyr Pro Leu Gln Ser Phe Tyr Ser Met Met Asn Leu
 885 890 895
 Val Asp Ser Gln Asp Thr Met Arg Ile Leu Thr Ile Leu Glu Asn Ala
 900 905 910
 Pro Gln Pro Gly Asp Leu Ser Ala Leu Gln Gln Asp Glu Tyr Arg Pro
 915 920 925
 Ser Pro Ala Ala Glu Gln Leu Gly Ile Glu Arg Leu Lys Leu Val Ser
 930 935 940
 Asp Phe Gln Phe Ser Phe Pro Gly Asp Pro Thr Ile Phe Tyr Gly Asp
 945 950 955 960
 Glu Ala Gly Leu Thr Gly Tyr Ser Asp Pro Leu Asn Arg Arg Thr Tyr
 965 970 975
 Pro Trp Asp Asn Gln Asn Leu Asp Leu Leu Asn His Tyr Arg Lys Leu
 980 985 990
 Gly Ala Ile Arg Asn Ala Asn Pro Val Leu Gln Thr Gly Asp Phe Thr
 995 1000 1005
 Pro Leu Tyr Ala Gln Gly Met Val Tyr Ala Phe Ala Arg Thr Ile Arg
 1010 1015 1020
 Asn Gly Arg Asp Val Phe Gly Val Pro Ala Glu Asp Ala Thr Ala Ile
 1025 1030 1035 1040
 Val Ala Ile Asn Asn Gln Asn Gln Ala Ile Thr Val Thr Ile Pro Thr
 1045 1050 1055
 Asp Gly Thr Val Ala Asp Gly Ser Thr Met Leu Asp Glu Leu Asn Asn
 1060 1065 1070
 Gln Trp Tyr Lys Val Gln Asn Gly Gly Ile Thr Leu Thr Leu Gln Ser
 1075 1080 1085
 Tyr Gln Gly Ala Ile Leu Val Thr Pro Ser Asp Ala Pro Met Ala Tyr
 1090 1095 1100
 Leu Gln Glu Glu Asp Ser Gln Asn Glu Ile Ala Trp Thr Pro Val Gln
 1105 1110 1115 1120
 Gly Ala Ile Gly Tyr Arg Val Trp Arg Gln Asn Pro Asn Gly Gln Trp
 1125 1130 1135

-47-

Val Pro Phe Gly Pro Val Leu Pro Ala Thr Asp Leu Ser Val Thr Val
 1140 1145 1150
 Glu Arg Asp Ala Tyr Ala Gln Thr Phe Ala Val Gln Ala Leu Phe Ser
 1155 1160 1165
 Ala Ser Asp His Ala Gln Ser Pro Val Ser Ala Pro Lys Thr Val Ser
 1170 1175 1180
 Leu Pro Val Asp Val Pro Ala Val Arg Leu Ser Gln Pro Ile Val Ser
 1185 1190 1195 1200
 Gly Arg Val Val Gly Asp Arg Ala Met Val Ser Ile Thr Pro Val Ser
 1205 1210 1215
 Gly Ala Thr Gln Tyr Val Ile Tyr Gln Arg Gln Gly Asp Gly Ser Tyr
 1220 1225 1230
 Ala Pro Val Ala Thr Val Ser Thr Ser Gly Asp Ser Ala Ala Ile Gly
 1235 1240 1245
 Glu Val Pro Ala Gln Gly Pro Ala Asn Ser Pro His Ala Thr Ile Arg
 1250 1255 1260
 Val Thr Val Pro Val Pro Ala Gly Phe Ser Ser Val Thr Tyr Arg Val
 1265 1270 1275 1280
 Ala Ala Gln Asn Glu Asp Gly Gln Ala Val Thr Asn Pro Leu Thr Leu
 1285 1290 1295
 Ser Leu Ser Lys Lys
 1300

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 47 bases
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Double Stranded
 - (D) TOPOLOGY: Linear

(ii) MOLECULAR TYPE: Nucleic Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAGAAGATIG TIGGGTCIII IGGGAAGAG AACTGGAAGT CIGAIAC

47

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 32 bases
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Double Stranded
 - (D) TOPOLOGY: Linear

-48-

(ii) MOLECULAR TYPE: Nucleic Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAGTCCTGCT GIGTICCGAA ICCIGGGTCG AT

32

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 bases

(B) TYPE: Nucleic Acid

(C) **STRANDEDNESS:** Double Stranded

(D) **TOPOLOGY:** Linear

(ii) MOLECULAR TYPE: Nucleic Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCIATGTTIC CGTTITAGAA IGGGTCIGGCA TIATCTCITA IATACIGCG TG

52

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 Amino Acids

(B) TYPE: Amino Acid

(C) STRANDEDNESS:

(D) **TOPOLOGY:** Linear

(ii) MOLECULAR TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TYR ASP THR ALA ASP TYR PHE LYS

1

5

8

(2) INFORMATION FOR SEQ ID NO:7:

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH: 17 Amino Acids

(B) TYPE: Amino Acid

(C) **STRANDEDNESS:**

(D) **TOPOLOGY:** Linear

(ii) MOLECULAR TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

LEU VAL SER ASP PHE GLN PHE SER PHE PRO Xaa ASP PRO THR ILE PHE

1

5

10

15

TYR

17

-49-

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 Amino Acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ii) MOLECULAR TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ILE ASP PRO GLY PHE GLY THR GLN GLN ASP Xaa LEU ASN LEU VAL GLN
1 5 10 15
ALA
17

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 Amino Acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ii) MOLECULAR TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

HIS ALA VAL ILE TYR GLU ILE MET PRO ASP PRO PHE TYR ASN GLY ASN
1 5 10 15
ILE ALA
18

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 Amino Acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ii) MOLECULAR TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

LEU ASP TYR LEU LYS
1 5

-50-

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 Amino Acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ii) MOLECULAR TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

SER LEU GLY VAL ASN THR LEU TYR LEU MET PRO VAL PHE GLU ALA
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 Amino Acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ii) MOLECULAR TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

PHE GLY ASN PHE HIS SER ASN GLY
1 5 8

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 Amino Acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ii) MOLECULAR TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GLY ILE TYR VAL GLY ALA
1 5 6

-51-

WHAT IS CLAIMED IS:

1. Substantially pure acid α -amylase DNA.
2. The substantially pure acid α -amylase DNA of claim 1, wherein the sequence of said DNA is that of SEQ ID No. 1, or a biologically active fragment, variant, or functional derivative thereof.
3. A recombinant DNA molecule, wherein said recombinant DNA molecule comprises the DNA of claim 2.
4. A cloning vector, wherein said cloning vector comprises the recombinant DNA molecule of claim 3.
5. The cloning vector of claim 4, wherein said recombinant DNA molecule is capable of being expressed.
6. A host cell transformed with the recombinant DNA molecule of claim 3.
7. The host cell of claim 6, wherein said host cell is a bacterial host.
8. The host cell of claim 7, wherein said host cell is a *Lactobacillus* or a *Bacillus*.
9. The host cell of claim 8, wherein said host cell is *Lactobacillus*.

-52-

10. The host cell of claim 8, wherein said host cell is *Bacillus*.

11. A method for the production of acid α amylase, wherein said method comprises:

(i) providing a nucleic acid molecule comprising the nucleic acid seq of SEQ ID No. 1, or a fragment, variant or functional derivative thereof;

(ii) transforming a host with said molecule;

(iii) expressing said nucleic acid molecule in said host; and

(iv) isolating the acid α -amylase produced by said expression.

12. The method of claim 11, wherein said host is *Lactobacillus*.

13. The method of claim 12, wherein said host is *Bacillus*.

14. A substantially purified *B. acidocaldarius* acid α -amylase, wherein said acid α -amylase comprises the amino acid sequence of SEQ ID No. 2, or a biologically active fragment, variant or functional derivative thereof.

-53-

15. The substantially purified acid α -amylase of claim 14, wherein said acid α -amylase is produced by a method comprising:

(i) providing a nucleic acid molecule comprising the nucleic acid seq of SEQ ID No. 1, or a biologically active fragment, variant or functional derivative thereof;

(ii) transforming a host with said molecule;

(iii) expressing said nucleic acid molecule in said host; and

(iv) isolating the acid α -amylase produced by said expression.

16. A method for preserving feed or fodder, wherein said method comprises the addition of the host of claim 6 to said feed or fodder.

17. The method of claim 16, wherein said host is *Lactobacillus*.

18. The method of claim 16, wherein said feed is cereal grain.

1/8

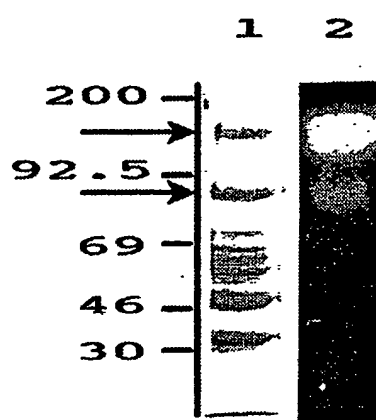


Fig. 1

2/8

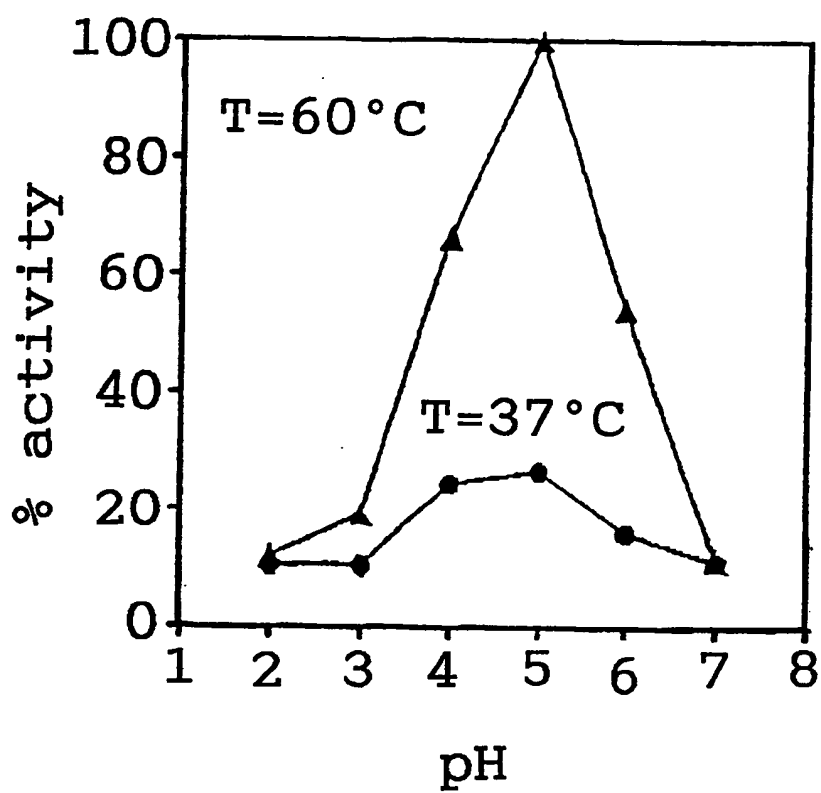


Fig. 2

SUBSTITUTE SHEET

3/8

Fig. 3 - page 1 of 6

B.Acidocaldarius amylase: DNA sequence

10	20	30	40	50	60
CATGTGGGTCGCCTCCTCTGCGATGGGACATCTCACTTACATTTTAACAGTGGAAGGGTG					
70	80	90	100	110	120
GGGAAGGGAACAAGGGTCTTGAGAGAAAAGAAAGGATTTCGGACAATGAACGATGCAAGAG					
130	140	150	160	170	180
CACCATTGACGTCGGGAAGGCTGAGCGTATAATCCAGGACGACAACGTTAGTGCAATCGT					
190	200	210	220	230	240
TTGCACAAGAGGAGGTTGATGCACGTGAAGTGGACGGGTGCCGCGAGCCTTGCGACGACC					
MetHisValLysTrpThrGlyAlaAlaSerLeuAlaThrThr					
250	260	270	280	290	300
ATCGTCATGTCTGCAGCGCTTCCGTTTCGCGGCGTTTGAAAACACCCGTTCCGTCGGGCACG					
IleValMetSerAlaAlaLeuProPheAlaAlaPheGluAsnThrArgSerValGlyThr					
310	320	330	340	350	360
GAACGCGTTCTGGCGGCGACCGCGGCGGATAGTACTAACGCCTCGCCCCCGACCGGGGCG					
GluArgValLeuAlaAlaThrAlaAlaAspSerThrAsnAlaSerProProThrGlyAla					
370	380	390	400	410	420
TCCGCAGGCGGTGGCACAGCAGGAGAGACCTACTCGAATGCCGTGCAGATTGTGAGCGGC					
SerAlaGlyGlyGlyThrAlaGlyGluThrTyrSerAsnAlaValGlnIleValSerGly					
430	440	450	460	470	480
AATGGCTTTCGCGCTGGAAACGGGGCCACGGTCGTCCTCGCTGTGAATGGACTGAACCTG					
AsnGlyPheArgAlaGlyAsnGlyAlaThrValValLeuAlaValAsnGlyLeuAsnLeu					
490	500	510	520	530	540
AACCCCTCGAGTTTCAAGGTTGTGGTATCGAATAGCCTCAACGGCGTGATCGACGTCACA					
AsnProSerSerPheLysValValValSerAsnSerLeuAsnGlyValIleAspValThr					
550	560	570	580	590	600
AGTGATTCCGTGCTAACGGGGCCGAATTCGATTGCCTTGACCTTCCTGCCGGGGATGAC					
SerAspSerValLeuThrGlyProAsnSerIleAlaLeuHisLeuProAlaGlyAspAsp					
610	620	630	640	650	660
GGGCTTGCGCCGGTACGTACACGGTCACCGTCGAAAGTGGAGGTGATTCCGGTCTCGACG					
GlyLeuGlyAlaGlyThrTyrThrValThrValGluSerGlyGlyAspSerValSerThr					
670	680	690	700	710	720
CCTTCAGGACAGGGACTTCAGGTCATGCCTTATACGACGGCCGACACCATTCACTGGGGAT					
ProSerGlyGlnGlyLeuGlnValMetProTyrThrThrAlaAspThrIleGlnTrpAsp					
730	740	750	760	770	780
GGAATCTACACATCCGATGGCGCGATGTACGTGTCAGATCCGAATCCCTCCCCTGGACAA					
GlyIleTyrThrSerAspGlyAlaMetTyrValSerAspProAsnProSerProGlyGln					

SUBSTITUTE SHEET

4/8

Fig. 3 - page 2 of 6

790 800 810 820 830 840
GAGGTGACGATCAGCCTTCGAGCGTACAGCGGGAACCTGACGAAGGTGATCCTGAATTGC
GluValThrIleSerLeuArgAlaTyrSerGlyAsnLeuThrLysValIleLeuAsnCys

850 860 870 880 890 900
TGGGACACCGCACAAACAAGGGTTTCCAGGTGAGATGTCTCCAGGCAGAACGTTTGGA
TrpAspThrAlaGlnAsnLysGlyPheGlnValGluMetSerProGlyArgThrPheGly

910 920 930 940 950 960
CCGTATCAGCTCTGGTCTGCCACGATCCCCGCTTCAAACGGCGGAACGATCTATTATCGC
ProTyrGlnLeuTrpSerAlaThrIleProAlaSerAsnGlyGlyThrIleTyrTyrArg

970 980 990 1000 1010 1020
TTTGACATATACGATGGCACCAGTTTTTCGCTGTCTCTCAGGTGACGGACTGCACACGTCT
PheAspIleTyrAspGlyThrSerPheAlaCysLeuSerGlyAspGlyLeuHisThrSer

1030 1040 1050 1060 1070 1080
GACGACATCAACAACAATTTCCCGTTGCCCGTGGGGACGGTCACGCTTTCGACACTTCAG
AspAspIleAsnAsnAsnPheProLeuProValGlyThrValThrLeuSerThrLeuGln

1090 1100 1110 1120 1130 1140
GCGAATCCCGGTGATACGGTGACGGTCTCCGACCCTGTAGGTGACTTCGCCGGAAGCCAG
AlaAsnProGlyAspThrValThrValSerAspProValGlyAspPheAlaGlySerGln

1150 1160 1170 1180 1190 1200
GATCAACCCAATCACACGGTGATACGGTTTGTCAACTCGTCGGGCGAAACGGCCGCCACA
AspGlnProAsnHisThrValIleArgPheValAsnSerSerGlyGluThrAlaAlaThr

1210 1220 1230 1240 1250 1260
GTCAATGGGACGAACGCGAGCTGGAACAGCGTGACGTTTACAGTCCCACAGAGCCTTCCA
ValAsnGlyThrAsnAlaSerTrpAsnSerValGlnPheThrValProGlnSerLeuPro

1270 1280 1290 1300 1310 1320
AACGGCTTGTATCGCGTCGAGATCGACACGGTCGCCAAGGACGCGGATGGGGTGGTCAAT
AsnGlyLeuTyrArgValGluIleAspThrValAlaLysAspAlaAspGlyValValAsn

1330 1340 1350 1360 1370 1380
GTCGAATTGGACAGGAGTGCGGAGCTTATTGTAGGGCCTCTGCCCGCGTGGATGCAAGCG
ValGluLeuAspArgSerAlaGluLeuIleValGlyProLeuProAlaTrpMetGlnAla

1390 1400 1410 1420 1430 1440
TATGCACATGATTTCGTTTCAGGCGTTCTACCGATCGCCTTTCGGAGCCGTGTCCACAGGA
TyrAlaHisAspSerPheGlnAlaPheTyrArgSerProPheGlyAlaValSerThrGly

1450 1460 1470 1480 1490 1500
ACCCCCATCACGCTTCGCCTGCGGGCTCCGCTCAGCGTGAAGAGTGCGACGCTTCGCCTC
ThrProIleThrLeuArgLeuArgAlaProLeuSerValLysSerAlaThrLeuArgLeu

1510 1520 1530 1540 1550 1560
TGGGGGGCAGCGGATCAGTCAGGCGAGATCGACCTGCCGATGCAGAAGCTCCAAATGTCG
TrpGlyAlaAlaAspGlnSerGlyGluIleAspLeuProMetGlnLysLeuGlnMetSer

SUBSTITUTE SHEET

5/8

Fig. 3 - page 3 of 6

1570 1580 1590 1600 1610 1620
GGAGACGAGTTGGCGCAACAAACCGGCGTGACGACATCAACGACTACACGTGGTGGACG
GlyAspGluLeuAlaGlnGlnThrGlyValGlnAspIleAsnAspTyrThrTrpTrpThr

1630 1640 1650 1660 1670 1680
GTGACCATCCCTGCGGCGGATGTGACCACACCCGGGACGATGTGGTATCAGTTCGTGACG
ValThrIleProAlaAlaAspValThrThrProGlyThrMetTrpTyrGlnPheValThr

1690 1700 1710 1720 1730 1740
GAGACGGACACTGGCCAGGTGGTCTACTACGATGACAATGGAGCTCAGCTTGAAGGGCCT
GluThrAspThrGlyGlnValValTyrTyrAspAspAsnGlyAlaGlnLeuGluGlyPro

1750 1760 1770 1780 1790 1800
GGCCAGGTTGGGTTGTCTTCCGACGGACCGAGCTACCAGATCAGCGTATACGAACGGGGA
GlyGlnValGlyLeuSerSerAspGlyProSerTyrGlnIleSerValTyrGluArgGly

1810 1820 1830 1840 1850 1860
TTTCAGACGCCAGATTGGCTGAAGCACGCCGTGATCTACGAAATCATGCCGGATCGGTTC
PheGlnThrProAspTrpLeuLysHisAlaValIleTyrGluIleMetProAspArgPhe

1870 1880 1890 1900 1910 1920
TACAATGGCAATATCGCCACGGAGGAGAATCCGAATACGCAAAGGGGATTTATGTAGGG
TyrAsnGlyAsnIleAlaThrGluGluAsnProAsnThrGlnLysGlyIleTyrValGly

1930 1940 1950 1960 1970 1980
GCCGATGGAACGGAGTCATTAGGCCCCATCCAGTTCACGAGAACTGGGACTCGCCGCC
AlaAspGlyThrGluSerLeuGlyProIleGlnPheHisGluAsnTrpAspSerProPro

1990 2000 2010 2020 2030 2040
TATGATCCGAATATTCCTCCGTTATCTGATCCCAAATTGCCAGTCTGCGAGGCAATGGC
TyrAspProAsnIleProProLeuSerAspProLysIleAlaSerLeuArgGlyAsnGly

2050 2060 2070 2080 2090 2100
CAATGGAACATTGACTTTTTTCGGAGGTGATTTGCAGGGCATCGAGGATAAGCTGGACTAC
GlnTrpAsnIleAspPhePheGlyGlyAspLeuGlnGlyIleGluAspLysLeuAspTyr

2110 2120 2130 2140 2150 2160
CTGAAGAGCCTTGGAGTCAATACGCTGTATCTGATGCCCCGTCTTTGAGGCGGAATCCAAT
LeuLysSerLeuGlyValAsnThrLeuTyrLeuMetProValPheGluAlaGluSerAsn

2170 2180 2190 2200 2210 2220
CACAAATATGACACAGCCGACTATTTCAAGATTGACCCTGGATTTGGAACGCAGCAGGAC
HisLysTyrAspThrAlaAspTyrPheLysIleAspProGlyPheGlyThrGlnGlnAsp

2230 2240 2250 2260 2270 2280
TGGCTGAATCTCGTACAGGCTGCGCACGCGAAGGGGTTCATATCATTCTCGACGGGGTG
TrpLeuAsnLeuValGlnAlaAlaHisAlaLysGlyPheHisIleIleLeuAspGlyVal

2290 2300 2310 2320 2330 2340
TTCGAAGATACCGGAAGTGACAGCGTATATTTCAACAAGTTCGGGAACCTCCACTCCAAC
PheGluAspThrGlySerAspSerValTyrPheAsnLysPheGlyAsnPheHisSerAsn

SUBSTITUTE SHEET

6/8

Fig. 3 - page 4 of 6

2350 2360 2370 2380 2390 2400
GGTGCCTGGCAGGCGTACCTGAAGAACCAGCCGTCGCTGTCGCCCTACTACTCGTGGTAC
GlyAlaTrpGlnAlaTyrLeuLysAsnGlnProSerLeuSerProTyrTyrSerTrpTyr

2410 2420 2430 2440 2450 2460
GTGTGGACAGGGAACACCTCAAACCCATACGATTCTGTGGTTTCAGATCGACACGCTGCCA
ValTrpThrGlyAsnThrSerAsnProTyrAspSerTrpPheGlnIleAspThrLeuPro

2470 2480 2490 2500 2510 2520
CTTACGGACACGTCGAACCCCGCCTATCAGCGATTCTGTATGGGAGCGACAACCTCAGTC
LeuThrAspThrSerAsnProAlaTyrGlnArgPheValTyrGlySerAspAsnSerVal

2530 2540 2550 2560 2570 2580
GCGCGTGTGTGGATCCGGAAGGTGCGGACGGATGGCGCTTGGACTCGGCCGACAACGGG
AlaArgValTrpIleArgGluGlyAlaAspGlyTrpArgLeuAspSerAlaAspAsnGly

2590 2600 2610 2620 2630 2640
AATTTCAACACGGCATGGTGGGGTGGCTTTTCGGCAGGCCGTGAAATCGATCGATCCCAAC
AsnPheAsnThrAlaTrpTrpGlyGlyPheArgGlnAlaValLysSerIleAspProAsn

2650 2660 2670 2680 2690 2700
GCAGCGATCATCGGCGAGATCTGGGACAATGCGACGAATGACAATGGAACGGATTGGTTG
AlaAlaIleIleGlyGluIleTrpAspAsnAlaThrAsnAspAsnGlyThrAspTrpLeu

2710 2720 2730 2740 2750 2760
ACGGGATCGACCTTCGACAGTGTAATGAACTACCAGTTCCGGAACGCCGTGATCGACTTC
ThrGlySerThrPheAspSerValMetAsnTyrGlnPheArgAsnAlaValIleAspPhe

2770 2780 2790 2800 2810 2820
TTCCGCGGCACGTACAACGACGGAAACGTGCAGCACCACGCCGTGACGCTGCGGGATTC
PheArgGlyThrTyrAsnAspGlyAsnValGlnHisHisAlaValAspAlaAlaGlyPhe

2830 2840 2850 2860 2870 2880
AACCAGGAAGTATGCGCCTGTACAGTGAATATCCTCTGCAGTCGTTCTACTCGATGATG
AsnGlnGluLeuMetArgLeuTyrSerGluTyrProLeuGlnSerPheTyrSerMetMet

2890 2900 2910 2920 2930 2940
AACCTTGTCGATTTCGCAAGACACCATGCGGATCCTGACCATCTTGGAGAACGCGCCGAG
AsnLeuValAspSerGlnAspThrMetArgIleLeuThrIleLeuGluAsnAlaProGln

2950 2960 2970 2980 2990 3000
CCAGGCGATCTATCCGCGCTCCAGCAGGATGAGTACAGGCCGTCTCCTGCGGCTGAACAG
ProGlyAspLeuSerAlaLeuGlnGlnAspGluTyrArgProSerProAlaAlaGluGln

3010 3020 3030 3040 3050 3060
TTGGGGATCGAGAGGCTGAAGCTTGTATCGGACTTTCAATTCAGCTTCCCGGGCGATCCG
LeuGlyIleGluArgLeuLysLeuValSerAspPheGlnPheSerPheProGlyAspPro

3070 3080 3090 3100 3110 3120
ACCATCTTCTACGGCGACGAGGCAGGGCTCACTGGTTATTCGGATCCCCTCAATCGTCGG
ThrIlePheTyrGlyAspGluAlaGlyLeuThrGlyTyrSerAspProLeuAsnArgArg

SUBSTITUTE SHEET

Fig. 3 - page 5 of 6

3130 3140 3150 3160 3170 3180
ACCTATCCGTGGGACAACCAGAATCTCGATCTCCTGAACCACTACCGCAAGCTCGGGGCC
ThrTyrProTrpAspAsnGlnAsnLeuAspLeuLeuAsnHisTyrArgLysLeuGlyAla

3190 3200 3210 3220 3230 3240
ATTGAAACGCCAATCCTGTGCTTCAGACGGGGGATTTCACGCCGTTGTACGCACAGGGC
IleArgAsnAlaAsnProValLeuGlnThrGlyAspPheThrProLeuTyrAlaGlnGly

3250 3260 3270 3280 3290 3300
ATGGTGACGCATTTGCAAGGACCATTTCGGAATGGGCGAGATGTCTTCGGTGTGCCAGCG
MetValTyrAlaPheAlaArgThrIleArgAsnGlyArgAspValPheGlyValProAla

3310 3320 3330 3340 3350 3360
GAGGATGCCACGGCCATTGTGGCGATCAACAATCAGAACCAAGCTATCACCGTGACCATT
GluAspAlaThrAlaIleValAlaIleAsnAsnGlnAsnGlnAlaIleThrValThrIle

3370 3380 3390 3400 3410 3420
CCGACGGATGGGACGGTTGCGGACGGGTCCACGATGCTCGATGAACTGAACAACCAGTGG
ProThrAspGlyThrValAlaAspGlySerThrMetLeuAspGluLeuAsnAsnGlnTrp

3430 3440 3450 3460 3470 3480
TACAAGGTGCAGAAATGGTGGCATTACACTCACGCTGCAATCGTATCAAGGTGCCATTTTG
TyrLysValGlnAsnGlyGlyIleThrLeuThrLeuGlnSerTyrGlnGlyAlaIleLeu

3490 3500 3510 3520 3530 3540
GTGACGCCGAGCGACGCGCCGATGGCTTATCTGCAAGAGGAGGATTCTCAGAACGAGATT
ValThrProSerAspAlaProMetAlaTyrLeuGlnGluGluAspSerGlnAsnGluIle

3550 3560 3570 3580 3590 3600
GCGTGGACGCCTGTGCAAGGTGCCATCGGTTATCGCGTCTGGAGACAGAATCCGAATGGA
AlaTrpThrProValGlnGlyAlaIleGlyTyrArgValTrpArgGlnAsnProAsnGly

3610 3620 3630 3640 3650 3660
CAATGGGTGCCCTTTGGACCTGTGCTTCCTGCCACGGACTTGAGTGTACGGTGGAAACGC
GlnTrpValProPheGlyProValLeuProAlaThrAspLeuSerValThrValGluArg

3670 3680 3690 3700 3710 3720
GATGCATATGCGCAAACGTTTGCTGTACAAGCGCTGTTTTTCGGCGTCTGATCACGCCCAG
AspAlaTyrAlaGlnThrPheAlaValGlnAlaLeuPheSerAlaSerAspHisAlaGln

3730 3740 3750 3760 3770 3780
TCTCCGGTGTCCGGCACCTAAGACGGTATCGCTTCCCGTCGATGTGCCCCGGGTACGCCTG
SerProValSerAlaProLysThrValSerLeuProValAspValProAlaValArgLeu

3790 3800 3810 3820 3830 3840
AGTCAGCCGATCGTTAGTGGTTCGTGTGGTTGGAGATCGTGCGATGGTCTCGATCACGCCG
SerGlnProIleValSerGlyArgValValGlyAspArgAlaMetValSerIleThrPro

3850 3860 3870 3880 3890 3900
GTTTCAGGCGCGACGCAGTATGTGATCTACCAGAGACAGGGCGACGGATCGTATGCTCCG
ValSerGlyAlaThrGlnTyrValIleTyrGlnArgGlnGlyAspGlySerTyrAlaPro

SUBSTITUTE SHEET

8/8

Fig. 3 - page 6 of 6

3910	3920	3930	3940	3950	3960
GTCGCGACGGTCTCCACAAGTGGCGATTCCGCAGCTATAGGGGAAGTTCCTGCGCAAGGT					
ValAlaThrValSerThrSerGlyAspSerAlaAlaIleGlyGluValProAlaGlnGly					
3970	3980	3990	4000	4010	4020
CCGGCCAACTCGCCTCACGCGACGATTTCGCGTGACAGTGCCCGTACCTGCAGGTTTCTCG					
ProAlaAsnSerProHisAlaThrIleArgValThrValProValProAlaGlyPheSer					
4030	4040	4050	4060	4070	4080
TCGGTGACCTACCGCGTGGCTGCGCAAAACGAAGATGGGCAAGCTGTGACCAATCCATTG					
SerValThrTyrArgValAlaAlaGlnAsnGluAspGlyGlnAlaValThrAsnProLeu					
4090	4100	4110	4120	4130	4140
ACCCTATCGCTCTCGAAAAAGTGATGCCTCGCGAAAAGGGCTATCGGAATTTTTCGAGA					
ThrLeuSerLeuSerLysLys---					

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/FI 92/00138

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5	C12N15/56; C12N1/21; C12N9/28;	A23K3/03
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP,A,0 410 498 (GIST-BROCADES) 30 January 1991 see the whole document ---	1
X	AGRICULTURAL AND BIOLOGICAL CHEMISTRY. vol. 50, no. 1, 1986, TOKYO JP pages 23 - 31; M. KANNO: 'A <i>Bacillus acidocaldarius</i> alpha-amylase that is highly stable to heat under acidic conditions' cited in the application see the whole document ---	1
X	STARCH vol. 31, no. 5, 1979, WEINHEIM DE pages 166 - 171; E. BOYER ET AL: 'Isolation and characterization of unusual bacterial amylases' cited in the application see the whole document --- -/-	1
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
29 JULY 1992	05.08.92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	VAN DER SCHAAL C.A.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8 901 970 (PLANT GENETIC SYSTEMS) 9 March 1989 see examples 1,2,7-25 ---	1-9,11, 12,14-18
A	GENE. vol. 19, 1982, AMSTERDAM NL pages 81 - 87; I. PALVA: 'Molecular cloning of alpha-amylase gene from Bacillus amyloliquefaciens and its expression in B. subtilis' see the whole document ---	1-8,10, 11,13-15

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. FI 9200138
SA 59061**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 29/07/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0410498	30-01-91	AU-A- 5953890	17-01-91
		WO-A- 9100353	10-01-91
		JP-T- 4500756	13-02-92
WO-A-8901970	09-03-89	EP-A- 0311469	12-04-89
		JP-T- 2501266	10-05-90